0888-8809/02/$15.00/0
Printed in U.S.A.
Molecular Endocrinology 16(2):342–352

PI3K Is Involved in the IGF-I Inhibition of TSH-Induced Sodium/Iodide Symporter Gene Expression

BIBIAN GARCÍA AND PILAR SANTISTEBAN

Instituto de Investigaciones Biomédicas "Alberto Sols," Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Madrid, Spain E-28029

Here we studied the role of IGF-I on the regulation of the sodium/iodide symporter (NIS) gene expression in FRTL-5 thyroid cells. IGF-I did not modify NIS mRNA levels but inhibited TSH- and forskolin-induced NIS mRNA expression in a dose-dependent manner. We explored the signaling pathways by which IGF-I mediates the repression of NIS expression. Inhibition of either the MAPK kinase or PKC activities had no effect. Interestingly, inhibition of PI3K blocked IGF-I repression of TSH-induced NIS mRNA and protein levels. This effect takes place at the transcriptional level, as IGF-I inhibited TSH-induced transcription of a luciferase reporter construct containing a 2.8-kb DNA fragment of the rat NIS promoter. The inhibitory effect of IGF-I on the NIS promoter was blocked by the PI3K inhibitor LY294002 and was mimicked by overexpression of a vector harboring the constitutively activated catalytic subunit of PI3K. Using internal deletions of the NIS promoter, we defined a region from −1,947 to −1,152 responsible for the observed IGF-I/PI3K inhibitory effect. When fused to a heterologous promoter, this region inhibits transcription in response to IGF-I. These results demonstrate a central role for PI3K in the repression of NIS gene transcription by IGF-I and suggest the existence, within the above defined promoter region, of putative PI3K-responsive elements. (*Molecular Endocrinology* 16: 342–352, 2002)

IODIDE UPTAKE IS the first step in thyroid hormone biosynthesis (1), the primary function of the thyroid gland. Iodide transport occurs across the basolateral membrane of thyroid follicular cells in an active transport process mediated by the sodium iodide symporter (NIS) (2). Iodide is subsequently incorporated by thyroid peroxidase (TPO) into the thyroglobulin (Tg) molecule. All of these steps are stimulated by TSH, the primary hormone regulating the functions of differentiated thyroid cells. Although TSH regulation of iodide transport has been widely reported (3–5), the cloning of the rat NIS cDNA by Dai *et al.* (2) allowed the study of the molecular mechanisms involved in TSH regulation of NIS gene expression. TSH, via cAMP, induces the activity of the NIS protein (6, 7) and increases NIS mRNA levels (8). TSH stimulation of NIS mRNA expression in FRTL-5 cells is inhibited by RA (9), TNFα, ceramide, TGFβ1, and aging (10). The cloning of the rat NIS promoter (11, 12) allowed initiation of the study of NIS transcriptional regulation. A TSH-responsive element was identified between positions −420 and −385 (13) and a thyroid transcription factor-1 (TTF-1) binding site was identified between −245 and −230 (14). In the distal promoter, Ohno *et al*. (12) identified a short enhancer, called NUE (NIS upstream enhancer), another important regulatory element for TSH-regulated transcriptional activation. This element contains binding sites for the thyroid-specific transcription factors TTF-1 and Pax-8, and a cAMP responsive element-like sequence. NUE confers thyroid-specific transcriptional activity to the NIS gene and responds to TSH/cAMP. The cAMP response therefore requires the binding of Pax-8 and the integrity of the cAMP-responsive element sequence.

Insulin and IGF-I are additional important factors for thyroid function that collaborate with TSH in the regulation of thyroid proliferation and differentiation. The biological effects of insulin and IGF-I are mediated by activation of their cell surface receptors, which possess tyrosine kinase activity (15). After ligand binding, insulin and IGF-I receptor activities converge on the phosphorylation of insulin receptor substrates (IRS-1 and IRS-2), which then act as docking proteins for SH2-containing proteins such as PI3K. The interaction of the PI3K-regulatory subunit p85 with tyrosine-phosphorylated IRS-1 leads to activation of the p110 catalytic subunit. This results in activation of several protein kinases, including Akt and certain PKC isoforms. Grb-2 is another IRS-1-associated protein; their interaction leads to activation of the small GTP-binding protein Ras, which then initiates the Raf-1/MAPK kinase (MEK)/MAPKs ERK1 and ERK2 cascade. Stimulation of any of these pathways can lead to activation/inhibition of target genes by transcriptional or posttranscriptional mechanisms (16). Although the insulin/IGF-I signaling pathways involved in the regulation of thyroid-specific genes remain unknown, several reports have shown their importance in regulating thy-

Abbreviations: BIS, Bisindolyl maleimide I; GEF, guanine-nucleotide-exchange factor; IRS, insulin receptor substrate; MEK, MAPK kinase; NIS, sodium/iodide symporter; NUE, NIS upstream enhancer; PDBu, phorbol-12,13-dibutyrate; PKD, protein kinase D; RSV-CAT, Rous sarcoma virus-chloramphenicol acetyltransferase; Tg, thyroglobulin; TPO, thyroid peroxidase; TTF-1, TTF-2, thyroid transcription factors 1 and 2.

roid gene transcription. Insulin and IGF-I stimulate Tg and TPO mRNA expression (17, 18). Both hormones increase Tg transcription, and their effects are additive to those of TSH/cAMP (17). Our group has identified response elements for insulin and IGF-I within the Tg (19) and TPO (20) promoters and reported that the hormonal regulation of both genes is mediated mainly by the forkhead thyroid transcription factor-2 (TTF-2), a thyroid-specific transcription factor that binds to the promoter of both genes (21, 22). In addition, TTF-2 gene expression is stimulated by cAMP and insulin/IGF-I (23). Insulin and IGF-I also stimulate transcription of the TSH receptor gene (24).

Here we studied the role of IGF-I in the regulation of NIS gene expression and the signaling pathways involved in IGF-I action. The results show that IGF-I inhibits TSH/forskolin-induced NIS mRNA levels. This effect is PI3K mediated, as its specific inhibitor, LY294002, prevents IGF-I inhibition of TSH induction; this effect was also observed when NIS protein levels were determined. Using transient transfection assays, we show that IGF-I inhibits TSH-stimulated transcriptional activity of a 2.8-kb NIS promoter, but no inhibition was observed using constructs with internal deletions from −1,947 to −1,152. When fused to a heterologous promoter, this region inhibits transcription in response to IGF-I, indicating that it contains IGF-I-responsive elements. Presence of the PI3K inhibitor LY294002 prevents IGF-I inhibition, and cotransfection with a constitutively active form of the PI3K catalytic subunit reduces TSH-dependent transcriptional activation. Our results show that via PI3K activation, IGF-I interferes with TSH induction of NIS gene expression; we define a region within the promoter responsible for the IGF-I inhibitory effect.

RESULTS

Insulin and IGF-I Do Not Increase NIS mRNA Levels

Insulin and IGF-I are important hormones for thyroid function; both stimulate expression of the thyroid-specific genes Tg and TPO (17, 18), as well as the transcription factor TTF-2 (23), and potentiate the stimulatory action of TSH on the expression of these genes. We performed Northern hybridization assays to examine the role of insulin and IGF-I in NIS gene expression. Starved FRTL-5 cells were insulin or IGF-I treated, and NIS mRNA levels were compared with those observed in untreated cells or cells treated with TSH or forskolin. TSH and forskolin stimulate NIS expression, as has been extensively described (8), but neither IGF-I nor insulin has any effect on NIS mRNA levels compared with untreated cells (Fig. 1).

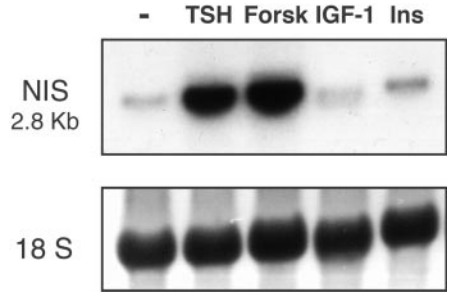

Fig. 1. IGF-I and Insulin Do Not Increase NIS mRNA Levels

Starved FRTL-5 cells were treated with TSH, forskolin (Forsk), IGF-I, or insulin (Ins) for 24 h and then harvested for RNA extraction. A representative Northern blot hybridized with the rat NIS probe is shown. Methylene blue staining of the 18S rRNA shows equal sample loading.

IGF-I Inhibits TSH- or Forskolin-Induced NIS mRNA Levels

Although neither insulin nor IGF-I increased NIS mRNA content in FRTL-5 cells, we analyzed whether these hormones could regulate TSH and forskolin effects. Starved FRTL-5 cells were treated with TSH (1 nM) or forskolin (10 μM), alone or in the presence of increasing concentrations of IGF-I. The results show that IGF-I inhibited the stimulatory effect of TSH or forskolin in a dose-dependent manner (Fig. 2, A and B, respectively). Maximum inhibition of NIS mRNA levels was reached at 100 ng/ml of IGF-I. Like IGF-I, insulin inhibited NIS mRNA expression induced by TSH or forskolin in a dose-dependent fashion; maximum inhibition was observed at 10 μM insulin (not shown). These data suggested that insulin and IGF-I may interfere with cAMP induction of NIS mRNA expression.

Neither MEK nor PKC Is Involved in IGF-I-Dependent Inhibition of TSH-Stimulated NIS mRNA Expression

To further analyze the mechanism by which IGF-I inhibits TSH-induced NIS mRNA expression, we studied the signaling pathways involved in IGF-I action. After binding of IGF-I to its cell surface receptors, a signaling cascade is initiated, leading to the activation of several kinases including MEK and PKC. To assess the involvement of these kinases in the IGF-I effect, we treated starved FRTL-5 cells with the specific inhibitors of these kinases, PD98059, which inhibits MEK activity, and bisindolyl maleimide I (BIS), which inhibits PKC activity, and determined NIS mRNA levels in response to TSH or TSH plus IGF-I. Figure 3A shows the results obtained with these inhibitors as well as with H89, a specific inhibitor of PKA. TSH induction of NIS mRNA expression is only partially inhibited in the presence of H89. This confirms previous observations by Ohno *et al*. (12) suggesting that TSH induction of NIS gene expression occurs in both a PKA-dependent and -independent manner. TSH retained the ability to in-

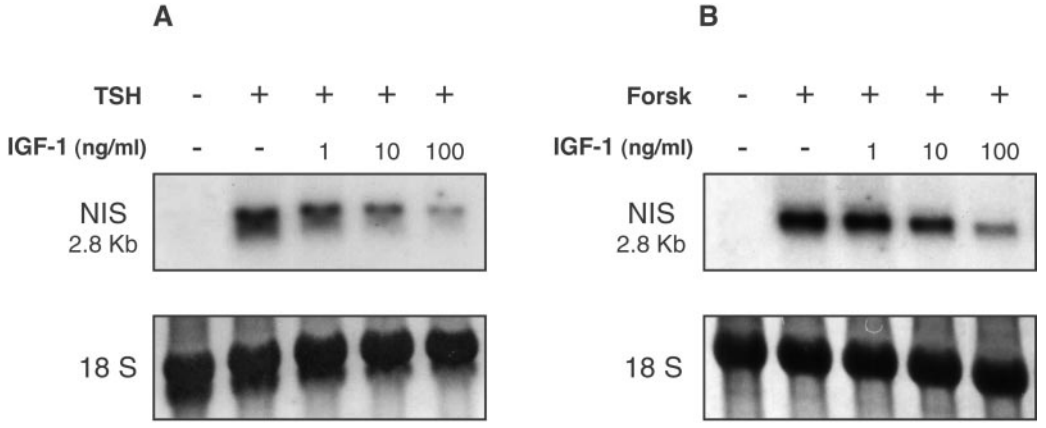

Fig. 2. IGF-I Inhibits TSH- or Forskolin-Stimulated NIS mRNA Expression

Starved FRTL-5 cells were treated with TSH (panel A) or forskolin (Forsk; panel B) alone or in the presence of increasing IGF-I concentrations (1, 10, or 100 ng/ml) for 24 h and then harvested for RNA analysis. A representative Northern blot hybridized with rat NIS probe is shown. Methylene blue staining of the 18S rRNA shows equal sample loading.

crease NIS mRNA levels in the presence of PD98059 or BIS. None of these inhibitors (PD98059 or BIS) abolished IGF-I inhibition of TSH induction, indicating that neither MEK nor PKC is involved in IGF-I action. The inhibitors have no effect when added alone and do not substantially affect mRNA levels in cells treated with IGF-I in the absence of TSH (not shown). The lack of any effect in response to PD98059 or BIS was not due to a loss of inhibitor function, demonstrated by immunoblot assays (Fig. 3B). Starved FRTL-5 cells treated with IGF-I show increased levels of phospho-ERK1/ERK2, downstream targets of MEK (25), compared with untreated cells; the presence of PD98059 abolished this stimulation (Fig. 3B, *upper panel*). BIS activity was examined by determining the activation of the PKC target, protein kinase D (PKD), using a specific antibody that recognizes phosphorylated PKD (26). The phorbol ester phorbol-12,13-dibutyrate (PDBu) stimulates PKD phosphorylation, but no phospho-PKD is detected in the presence of BIS (Fig. 3B, *lower panel*). These results clearly indicate that both inhibitors were active at the concentrations used.

Inhibition of PI3K Prevents Suppression of TSH-Induced NIS mRNA and Protein Expression by IGF-I

PI3K is one of the central enzymes implicated in insulin and IGF-I signaling. To determine whether PI3K is required for IGF-I inhibition of TSH induction of NIS gene expression, starved FRTL-5 cells were pretreated with the PI3K inhibitor LY294002 before the addition of TSH, forskolin, and IGF-I. LY294002 pretreatment increased NIS mRNA levels in TSH-treated cells and slightly enhanced the messenger level in cells treated with IGF-I (Fig. 4A). Interestingly, IGF-I inhibition of TSH induction is not observed in the presence of LY294002. Similar results were obtained using wortmannin (25–100 nM), another PI3K inhibitor (not shown). When forskolin was used instead of TSH, the results resembled those found for TSH, except that LY294002 did not increase NIS mRNA levels in forskolin-treated cells. These data suggest that PI3K interferes with NIS gene expression induced by the cAMP signaling pathway. We next determined whether the changes in NIS mRNA levels in response to TSH, IGF-I, and LY294002 correlated with changes in NIS protein levels. Western blot analyses were performed using a specific anti-NIS antibody that reacts with two main NIS species, the mature 80- to 90-kDa form and a partially processed 60- to 65-kDa NIS form (6). Due to the long half-life of the NIS protein, FRTL-5 cells were maintained for 7 d in starvation medium, before hormone addition, to completely deplete cells of NIS protein (7). Cells maintained for 7 d in starvation medium have no detectable NIS protein, and TSH treatment of cells led to an increase in NIS protein levels (Fig. 4C), as previously reported (6, 7). IGF-I completely inhibits NIS protein expression in response to TSH, and the presence of LY294002 prevents this effect. All together, the results suggest that PI3K interferes with TSH-dependent NIS mRNA and protein induction and may mediate the IGF-I inhibitory effect observed.

IGF-I Inhibits TSH-Dependent Transcriptional Activation of the NIS Gene

To study whether IGF-I regulates transcription of the rat NIS gene, we cloned a 2.8-kb fragment of the rat NIS promoter, as described in *Materials and Methods*. This region contains the NUE, a regulatory element necessary for a full TSH response (12). Luciferase reporter DNA constructs containing the full-length DNA fragment or 5′-deletion derivatives were transiently transfected into FRTL-5 cells and assayed for transcriptional activity in response to TSH and/or IGF-I (Fig. 5A). As previously described (12), the 2,854-bp

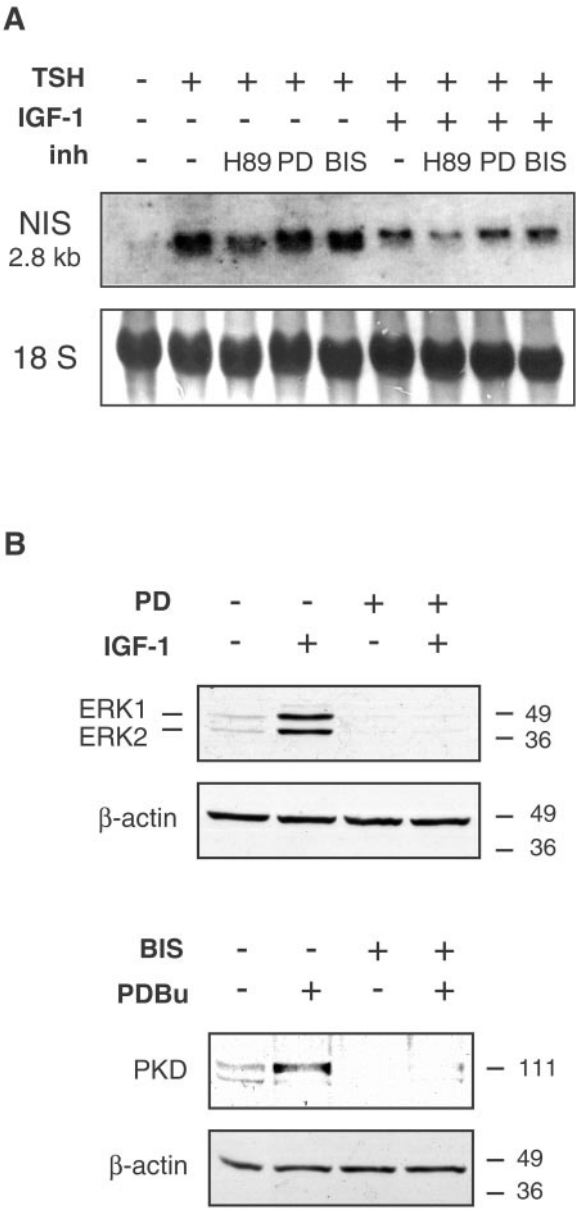

Fig. 3. The Inhibitors H89, PD98059, and BIS Do Not Block IGF-I Inhibition of TSH-Induced NIS mRNA Expression

A, Starved FRTL-5 cells were pretreated with the inhibitors H89, PD98059 (PD), or BIS, 30 min before addition of TSH and IGF-I. After 24 h, cells were harvested for RNA analysis. A representative Northern blot hybridized with rat NIS probe and methylene blue staining of the 18S rRNA are shown. B, Starved FRTL-5 cells were pretreated with inhibitors PD or BIS, 30 min before addition of IGF-I (100 ng/ml) or PDBu (100 nM) for 15 min. Western blot analyses performed with 20 μg of total protein and probed with antiphospho-ERK1/ERK2 (*upper panel*) and antiphospho-PKD (*lower panel*) are shown. Immunodetection of β-actin shows equal sample loading.

DNA fragment (pNIS-2.8) showed significant stimulation of transcription by TSH, whereas the 5′-deletion constructs, pNIS-2, pNIS-1.2, and pNIS-0.5, which lacked the enhancer, showed no stimulation of transcription or only trace levels, as was the case for pNIS-0.5. IGF-I had no effect on the transcriptional activity of any construct when added alone, although IGF-I inhibited the TSH-induced transcriptional activation of the construct containing the full promoter, pNIS-2.8; this indicates that IGF-I signaling interferes with TSH induction of NIS transcription. To analyze the mechanism of NIS repression by IGF-I, we inserted a region containing the NUE element upstream of the promoter region in pNIS-1.2 (pNIS-NUE-1.2) and pNIS-0.5 (pNIS-NUE-0.5). Transient transfection analyses with these two constructs and pNIS-2.8 showed that all three constructs expressed significant luciferase activity in response to TSH, but only pNIS-2.8 exhibited a reduction in luciferase activity when IGF-I was added together with TSH (Fig. 5B). These results indicate that IGF-I does not interfere with NUE activation by TSH and requires the region from −1,947 to −1,152 for the inhibition of the TSH-dependent stimulation of NIS transcription.

To confirm that this region is IGF-I responsive, the NIS-regulatory region from −1,947 to −1,152 was inserted upstream from the β-globin TATA box of the luciferase expression vector TATA-LUC (20). This construct (pNIS-0.8-TL) and the control vector TATA-LUC were transiently transfected into FRTL-5 cells and assayed for transcriptional activity in response to TSH and/or IGF-I. The results indicate that pNIS-0.8-TL shows almost 50% inhibition by IGF-I, both alone or in the presence of TSH, whereas no effect is observed on the TATA-LUC control vector, which lacked the regulatory region (Fig. 5C). These results clearly indicate that the −1,947 to −1,152 region of the rat NIS gene contains IGF-I-responsive regulatory elements.

PI3K Mediates the Inhibition of TSH-Induced NIS Promoter Activity by IGF-I

To investigate whether PI3K was required for the observed IGF-I inhibitory action on NIS promoter activity, we studied the effect of LY294002 on TSH-induced transcriptional activation of pNIS-2.8, alone or in the presence of IGF-I. The results (Fig. 6A) resemble those obtained in Northern blot assays, *i.e.* the PI3K inhibitor LY294002 completely blocked IGF-I repression of the transcriptional activation by TSH. These data indicate that PI3K activity is necessary for suppression of TSH-induced NIS gene expression by IGF-I. To ask whether PI3K activation inhibited TSH induction of NIS gene transcription, we next transfected FRTL-5 cells with the pNIS-2.8, pNIS-NUE-1.2, or pNIS-NUE-0.5 constructs, with or without an expression vector for the constitutively active PI3K catalytic subunit, p110-CAAX. Overexpression of p110-CAAX caused a striking reduction in the stimulation of pNIS-2.8 transcription by TSH, thus mimicking the effect of IGF-I (Fig. 6B). Conversely, the transcriptional activation of pNIS-NUE-1.2 and pNIS-NUE-0.5 by TSH is not affected by p110-CAAX overexpression. These results indicate that p110-CAAX does not block NUE activation by

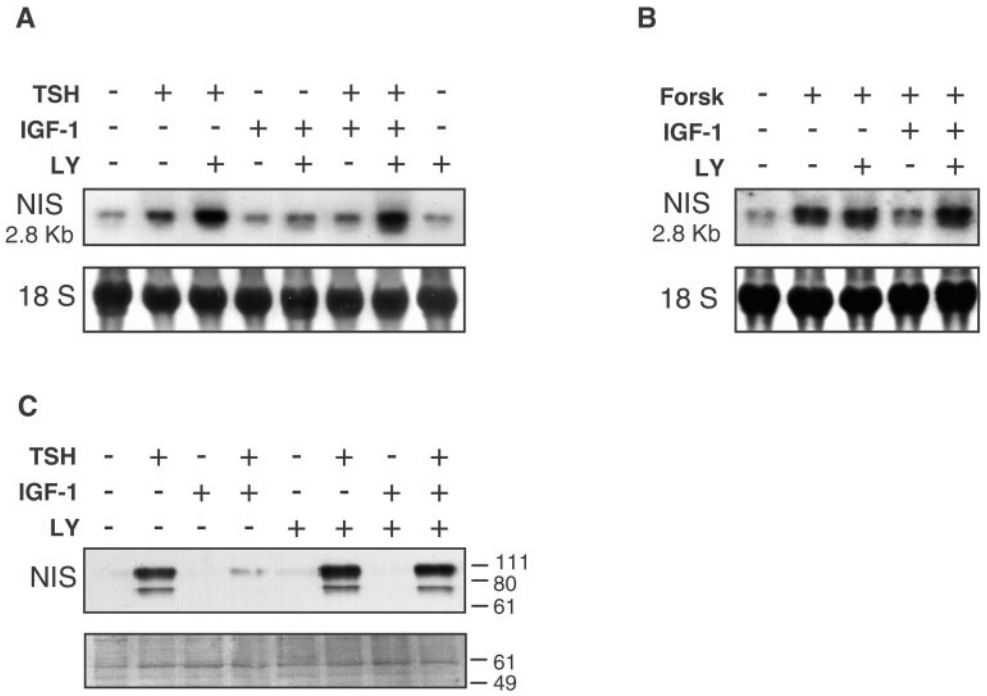

Fig. 4. The PI3K Inhibitor LY294002 Blocks IGF-I Inhibition of TSH- and Forskolin-Induced NIS mRNA and TSH-Induced Protein Expression

A and B, Starved FRTL-5 cells were pretreated with LY294002 (LY) 30 min before addition of TSH, forskolin (Forsk), or IGF-I, and cells were collected 24 h later. Representative Northern blots hybridized with rat NIS probe are shown. Methylene blue staining of the 18S rRNA shows equal sample loading. C, Western blot analysis of NIS expression. FRTL-5 cells were starved for 7 d and then pretreated with LY294002 (LY) 30 min before TSH and IGF-I addition. After 24 h, cells were collected for membrane protein preparation. Membrane protein (5 μg) was resolved in 12% SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-NIS antibody. For loading control, Ponceau S staining of a blot fragment is shown.

TSH, and that the region between −1,947 and −1,152 responds to PI3K activation, leading to inhibition of transcriptional activation by TSH.

DISCUSSION

The data presented here demonstrate that IGF-I inhibits TSH stimulation of NIS gene expression. These results are in accordance with previous reports showing that IGF-I inhibited iodide uptake stimulated by TSH in FRTL-5 cells (27, 28); this effect was also seen when the cAMP analog 8-Br-cAMP was used instead of TSH (28). We also demonstrate that IGF-I interferes with cAMP signaling, because IGF-I prevents the induction of NIS mRNA expression by forskolin. These results, in addition to the reports mentioned above (27, 28), thus demonstrate cross-talk between IGF-I signaling and the cAMP transduction pathway; this cross-talk may take place downstream of cAMP generation, because IGF-I did not affect the TSH-induced cAMP levels, as reported by Saji and Kohn (28).

We studied the signaling pathways involved in the IGF-I inhibitory effect on the induction of NIS gene expression by TSH. Using specific inhibitors of the distinct kinases involved in the most important signaling pathways operating in thyroid cells, we found that the presence of H89, a PKA inhibitor, decreased but did not abolish TSH induction of NIS mRNA levels. It is likely that cAMP regulation of NIS gene expression is mediated by PKA-dependent and -independent pathways, as suggested by Ohno *et al*. (12). A new cAMP cascade has been identified that involves the guanine nucleotide exchange factor (GEF), called Epac (29) or cAMP-GEF (30). Epac, which is regulated directly by cAMP, is a GEF for the small GTPase Rap-1 and is expressed in many tissues, including thyroid (30). In dog thyroid cells, Rap-1 activation has been described in response to TSH/cAMP (31) and, in the WRT rat thyroid cell line, TSH activates Rap1 through a cAMP-mediated, PKA-independent mechanism (32). It remains to be determined whether this cAMP-Epac-Rap-1 pathway is involved in TSH-dependent stimulation of NIS gene expression.

Contrary to observations with H89, the presence of the PI3K inhibitor LY294002 results in superinduction of NIS mRNA levels by TSH, suggesting a role for PI3K in TSH-dependent signaling transduction pathways. These data concur with recent results involving PI3K in the stimulation of thyroid cell proliferation in response

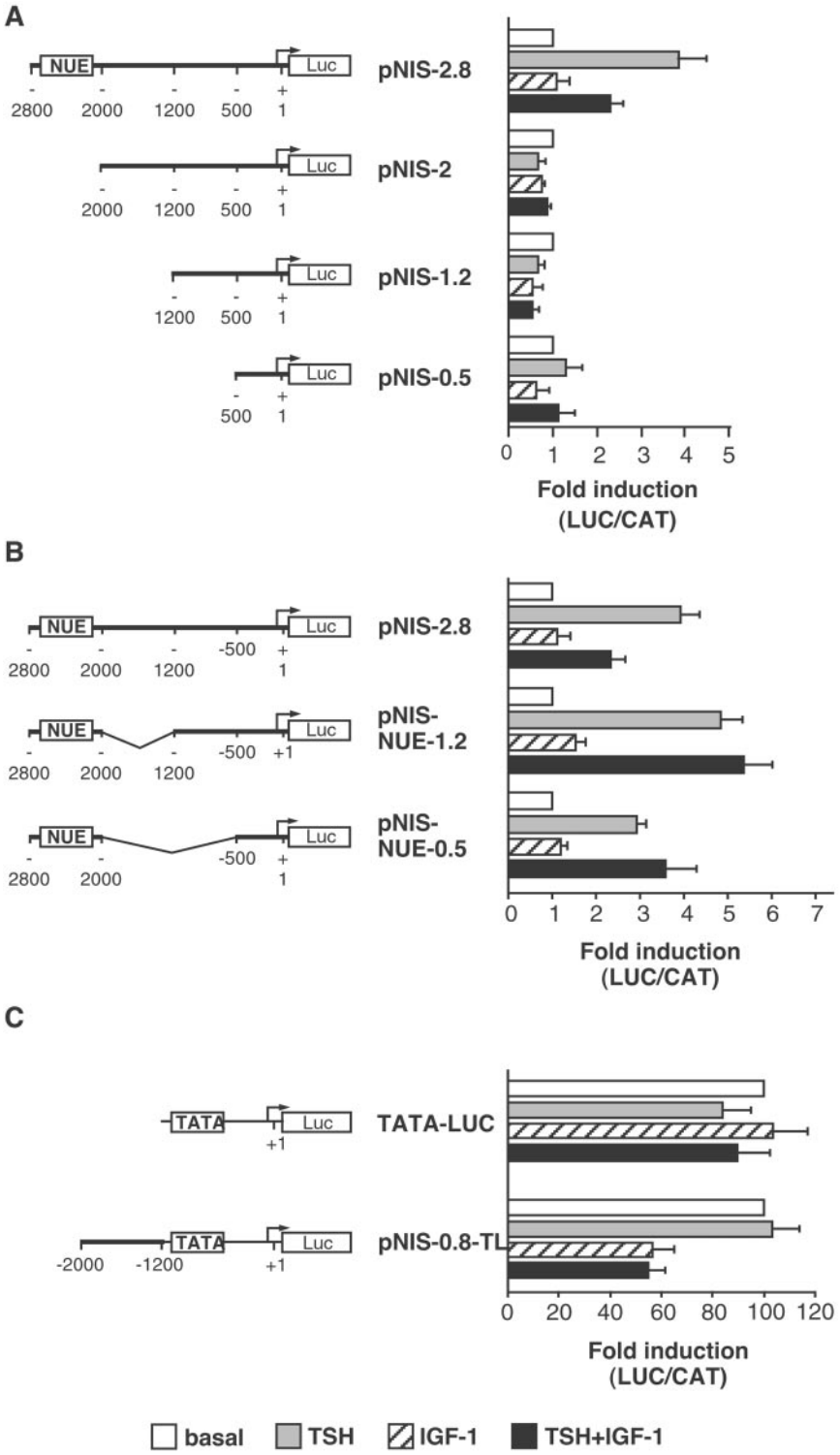

Fig. 5. IGF-I Inhibits TSH-Induced NIS Gene Transcription

A and B, FRTL-5 cells were transfected with 2 μg of RSV-CAT and 2 pmol of the reporter construct containing the complete NIS promoter, pNIS-2.8, or the 5′-deletion derivatives pNIS-2, pNIS-1.2, or pNIS-0.5 (panel A), or the internal deletion constructs pNIS-NUE-1.2 or pNIS-NUE-0.5 (panel B). C, FRTL-5 cells were transfected with 2 μg of RSV-CAT and 2 pmol of pNIS-0.8-TL or the control vector TATA-LUC. After transfection, cells were maintained 72 h in starvation medium and then treated with TSH and IGF-I. After 24 h, cells were harvested and luciferase and CAT activity were determined. Relative luciferase activity is the value of light units, normalizing the results to CAT activity derived from RSV-CAT. The NIS promoter activity is expressed as the x-fold induction over the basal levels (=1 in A and B; =100 in C) of starved cells (basal). The data represent the mean ± SE of at least four independent experiments.

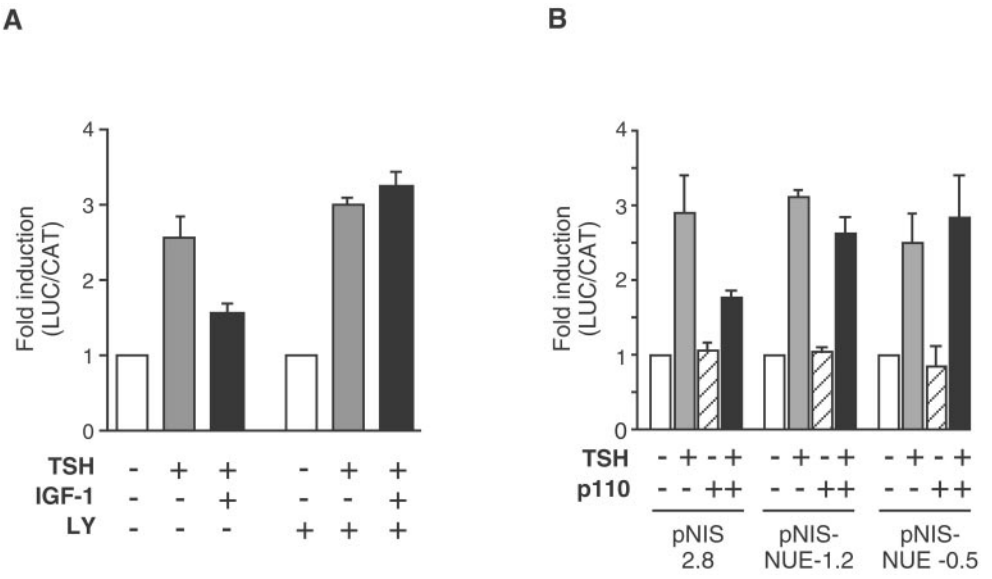

Fig. 6. PI3K Involvement in NIS Promoter Repression by IGF-I

A, FRTL-5 cells were transfected with 2 pmol of the reporter construct pNIS-2.8 and 2 μg of RSV-CAT. After 72 h in starvation medium, cells were treated with LY294002 (LY) 30 min before addition of TSH and IGF-I. B, FRTL-5 cells were transfected with 2 pmol of the reporter constructs pNIS-2.8, pNIS-NUE-1.2, or pNIS-NUE-0.5, and 2 μg of RSV-CAT, with 2 μg of either pSG5-p110-CAAX (p110) or the empty vector. After transfection, cells were maintained 72 h in starvation medium and then treated with TSH. At 24 h after hormone treatment, cells were harvested, and luciferase and CAT activity were determined. Relative luciferase activity was determined as in Fig. 5. NIS promoter activity is expressed as the x-fold induction over basal levels (=1) of starved cells. The data represent the mean ± SE of at least three independent experiments.

to TSH via cAMP (33, 34). PI3K signaling is thus required for a mitogenic response to TSH but, in view of our results, it inhibits the stimulation of NIS gene expression by TSH. Interestingly, we did not observe this superinduction when forskolin was used instead of TSH (Fig. 4). One explanation for this result may derive from the fact that forskolin stimulates cAMP production by direct activation of the adenylyl cyclase, thus eliminating possible PI3K activation by G protein $\beta\gamma$-subunits, a PI3K activation mechanism (35). Although it was not the aim of this study to analyze the signaling pathways involved in TSH-dependent activation of NIS gene expression, it would be of great interest to investigate the existence of this possible mechanism of PI3K activation in response to TSH.

IGF-I inhibition of TSH-stimulated NIS mRNA and protein expression was blocked by the PI3K inhibitor LY294002. We observed similar results with wortmannin at nanomolar doses (not shown). The absence of effect in response to PD98059 and BIS should not be attributed to a lack of function, as we observed an inhibitory effect by both compounds in Western blots for phospho-ERK1/ERK2 and phospho-PKD, respectively. The results clearly indicate that PI3K interferes with TSH-induced NIS gene expression and suggest that PI3K may be involved in IGF-I action. Previous data from Cass and Meinkoth (36) suggested a role for PI3K in the regulation of NIS expression. They reported that transfection of WRT thyroid cells with the Ras mutant RasV12C40, which signals preferentially through PI3K, resulted in a reduction of NIS protein levels, whereas Tg protein levels were not affected. It appears that NIS and Tg gene expression are regulated in a different manner. The authors proposed that RasV12C40-dependent signals might also downregulate the expression and/or activity of Pax-8, a transcription factor involved in the regulation of NIS expression (12), or affect NIS activity through a posttranscriptional mechanism. Before the cloning of the NIS cDNA (2), Saji and Kohn (28) proposed a posttranscriptional mechanism by which insulin/IGF-I regulated TSH induction of the iodide porter system. Although these results are not in doubt, an additional regulatory mechanism at the transcriptional level cannot be ruled out. Taking advantage of the cloning and characterization of the rat NIS promoter (11, 12), we cloned a 2.8-kb DNA fragment of the rat NIS promoter to study the role of IGF-I in regulating NIS gene transcription. Our results from transient transfection assays, with reporter constructs containing the full-length DNA fragment or 5′ deletions, confirm the requirement of the NUE for a potent TSH response, as reported previously (12). We also showed that transcriptional activation by TSH was inhibited by IGF-I, although the extent of inhibition was lower than that observed in Northern blot studies. This difference may be explained by the suggested posttranscriptional regulation (28) and/or the presence of regulatory elements upstream of the DNA fragment studied.

The results obtained with the PI3K inhibitor LY294002 indicated that PI3K activity was required for inhibition of TSH-induced transcriptional activation by

IGF-I. This observation was confirmed by the results of transient transfection assays with the constitutively active form of the PI3K catalytic p110α subunit, p110-CAAX, showing inhibition of transcriptional activation by TSH. Although there are multiple PI3K isoforms (37), these findings suggest that signaling pathways leading to activation of the PI3K p110α subunit could result in the inhibition of NIS gene expression. It nonetheless remains to be elucidated which PI3K subtype, sensitive to LY294002 and wortmannin inhibition, mediates IGF-I effects. To confirm the results observed with PI3K inhibitor, we performed transfection assays with the dominant negative form of the PI3K-regulatory subunit, p85$\alpha\Delta$iSH2-N. The results did not provide significant information, as transfection with this mutant resulted in the loss of the majority of the cells (not shown), *i.e.* drastic inhibition of PI3K activity leads to cell death. This concurs with recent reports on PI3K in cell cycle progression in thyroid cells (33, 34), in addition to its role in cell survival signaling (38). Added to our findings, these results clearly demonstrate the importance of PI3K in thyroid cell function. Although we have no physiological explanation for the IGF-I-inhibitory effect observed on NIS gene expression, the results with LY294002 and the dominant positive mutant of PI3K suggest that situations leading to activation of the PI3K pathway may affect NIS expression levels. In this respect, activation of Akt, one of the downstream targets of PI3K, has been shown in several thyroid tumors (39). Moreover, the expression level of PTEN, a 3′-phosphatase that converts phosphatidylinositol (3,4)P_2 to PtIns(4)P, and PtIns (3,4,5)P_3 to PtIns (4,5)P_2, thus inactivating Akt, is decreased in neoplasic thyroid cells and in thyroid tumors (40). These findings and our results may explain the downregulation of NIS expression found in many thyroid cancers (1). The elucidation of the mechanism by which PI3K downregulates NIS expression will aid in understanding the absence of iodide symporter activity in thyroid tumors.

To delimit the region within the NIS promoter responsible for the IGF-I effect, several deletions of the NIS promoter were studied. The results indicate that IGF-I does not interfere with the TSH activation of NUE. The region from $-1,947$ to $-1,152$ seems to be necessary for IGF-I/PI3K inhibition of TSH induction of the NIS promoter; it contains IGF-I-responsive elements, as it could inhibit transcription of a heterologous promoter in response to IGF-I. Computer analysis of this region revealed the existence of several TG/ATTT elements; this sequence is the core motif of insulin and IGF-I response elements found in the promoter of several genes that are negatively regulated by insulin/IGF-I (16). It has recently been reported that a subfamily of forkhead transcription factors, the FKHR proteins, can bind this motif and are targets of PI3K (41). This evidence suggests that these proteins, or related family members, may confer the inhibitory effect observed on NIS transcription. Additional observations by our group, indicating that the thyroid transcription factor TTF-2 mediates transcriptional activation of thyroid-specific genes by insulin/IGF-I (23), suggest a general role for forkhead proteins in mediating insulin/IGF-I effects on transcription. Further studies will elucidate the role of FKHR proteins on NIS gene expression and will give new insight into the mechanisms by which forkhead transcription factors regulate the transcription of cAMP-stimulated genes.

MATERIALS AND METHODS

Materials

Tissue culture medium, bovine TSH, and bovine insulin were purchased from Sigma (St. Louis, MO). IGF-I was obtained from Peprotech (Rocky Hill, NJ). Forskolin, LY294002, PD98059, H89, bisindolyl maleimide I HCl, and wortmannin were purchased from Calbiochem (La Jolla, CA). Donor calf serum and DMEM were obtained from Life Technologies, Inc. (Gaithersburg, MD), and Nytran and nitrocellulose filters were obtained from Schleicher & Schuell, Inc. (Keene, NH). The luciferase assay kit was purchased from Promega Corp. (Madison, WI). [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from ICN Biochemicals, Inc. Restriction enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN); the Luminol kit and streptavidin-horseradish peroxidase conjugate, antiphospho-ERK1/ERK2, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-NIS antibody was a generous gift from Dr. N. Carrasco (Albert Einstein College of Medicine, Bronx, NY). Anti-phospho-PKD antibody was kindly donated by Dr. T. Iglesias (Instituto de Investigaciones Biomédicas, Madrid, Spain).

Cell Culture

FRTL-5 cells (ATCC CRL 8305, American Type Culture Collection, Manassas, VA) were a generous gift of Dr. L. D. Kohn (Edison Biotechnical Institute, Athens, OH); these cells had the properties described (42, 43), were diploid, and their doubling time with TSH was 36 h. Cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% donor calf serum and a six-hormone mixture [1 nM TSH, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 nM hydrocortisone, and 10 ng/ml glycyl-L-histidyl-L-lysine acetate; complete medium (42)]. The effect of hormones and growth factors was studied by starving near-confluent cells for TSH and insulin in the presence of 0.2% serum (starvation medium) for 72 h. For NIS protein analyses, cells were starved for 7 d, after which the ligands were added to culture medium at the following concentrations: 1 nM TSH, 10 μg/ml insulin, 100 ng/ml IGF-I, 10 μM forskolin, and 100 nM PDBu (unless otherwise indicated). Inhibitors were added to the cells 30 min before hormone addition at the following concentrations: 10 μM H89, 50 μM PD98059, 25–100 nM wortmannin, 10 μM LY294002, and 1 μM bisindolyl maleimide I.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated by the guanidinium-thiocyanate-phenol procedure (44) from FRTL-5 cells after different treatments. Total RNA (20 μg) was separated in 1% agarose gels containing 2.2 M formaldehyde. RNA was blotted onto Nytran filters as suggested by the manufacturer. Methylene blue staining of the blots revealed the integrity of the RNA and the presence of equal amounts in each lane. Hybridization and washing were carried out with the NIS-specific probe (2), labeled by random oligo priming.

Protein Extraction and Western Blot Analysis

Membranes from FRTL-5 cells were prepared with protease inhibitors as described previously (45). Briefly, FRTL-5 cells were collected and homogenized in a buffer containing 250 mM sucrose, 10 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell homogenates were centrifuged (100,000 $\times$ *g*, 60 min, 4 C) to obtain membrane fractions, and the pellet was resuspended in buffer as above. Whole-cell extracts were obtained by resuspending the cell pellet in a buffer containing 50 mM HEPES (pH 7.0), 2 mM $MgCl_2$, 250 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP40, 1 mM dithiothreitol, 2 M Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Protein concentration was determined according to Bradford (46) with the Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay kit. For immunodetection of NIS, membrane fractions were diluted 1:2 with loading buffer and heated (37 C, 30 min) before electrophoresis. Total cell extract proteins (25 μg) or membrane proteins (5 μg) were separated by 8% or 12% SDS-PAGE for phospho-PKD or phospho-ERK1/ERK2 and NIS, respectively, and then transferred to nitrocellulose membrane. Ponceau S staining of the blots for NIS detection showed equal protein loading. Membranes were blocked (1 h, room temperature) in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% nonfat milk. Western analyses for NIS detection were performed with anti-NIS antibody (1:5,000 dilution) (45). PKD was detected using a noncommercial antibody to phosphorylated PKD (26). Commercial antibodies were used for ERK1/ERK2 or β-actin immunodetection at 1:5,000 or 1:2,000, respectively. After incubation with antibodies in TBS-T containing 5% nonfat milk, membranes were washed four times with TBS-T buffer and incubated with a streptavidin-horseradish peroxidase conjugate (1:2,000), followed by four washes of 10 min each with TBS-T buffer. Immunoreactive bands were visualized with the Luminol Western blot detection reagent (Santa Cruz Biotechnology).

Promoter Constructs

pNIS-2.8 and pNIS-2

DNA fragments from the rat NIS promoter, corresponding to the regions −2,841 to +13 and −1,941 to +13 (11, 12), were amplified from FRTL-5 genomic DNA by PCR, using forward primers with a *Bam*HI (5′-GCTATGGATCCCGAAGTGGCACTCACAACAT GTACC-3′) and *Sac*I sites (5′-TCCTGCGAGCTCTAAGCCTCTGCTAGG-3′), respectively, and reverse primer with a *Bam*HI site (5′-CGCAGGATCCATGGAGACAGGTGACTCG-3′). The fragments were cloned into pGEM-T vector (Promega Corp.), cleaved by *Bam*HI or *Sac*I and *Bam*HI, and subcloned into pBSLuc2 vector (22).

pNIS-1.2 and pNIS-0.5

DNA fragments were amplified from pNIS-2 by PCR, using forward primers with a *Sac*I site (5′-CAACACGA GCTCCAGCCCTCCCTGGTGGC-3′; 5′-AAGAAGAGCTCCAAGAGAACCTGAGTGC-3′) and the reverse primer described above.

pNIS-NUE-1.2 and pNIS-NUE-0.5

A DNA fragment containing the NUE element was cleaved by *Sac*I and inserted into the *Sac*I site of pNIS-1.2 (pNIS-NUE-1.2) and pNIS-0.5 (pNIS-NUE-0.5).

pNIS-0.8-TL

The DNA fragment was PCR-amplified from pNIS-2.8, using the forward primer with a *Sac*I site (5′-TCCTGCGAGCTCTAAGCCTCTGCTAGG-3′) and reverse primer with a *Bam*HI site (5′-ATGCTGGGATCCTCGCGGTCATGCCGGTGC-3′). The fragment was cloned into pGEM-T vector, cleaved by *Sac*I, and subcloned into the TATA-LUC vector (20). The fidelity of all constructs was confirmed by sequencing on an automatic DNA sequencer (Perkin-Elmer Corp., Norwalk, CT).

Transfection

FRTL-5 cells were plated at 1.5 $\times$ 10^6 cells per 90 mm-diameter tissue culture dish, 48 h before transfection. Transfections were performed by calcium phosphate coprecipitation as described previously (47). Cells were transfected with 2 pmol of test plasmid and 2 μg of Rous sarcoma virus-chloramphenicol acetyltransferase (RSV-CAT) (48), used to monitor transfection efficiency. PI3K pathway involvement was studied using an expression plasmid containing the constitutively active catalytic PI3K subunit, pSG5-p110-CAAX, or the dominant negative form, p85$\alpha\Delta$iSH2-N (49). To study the effect of pSG5-p110-CAAX on pNIS-2.8, pNIS-NUE-1.2, and pNIS-NUE-0.5, 2 μg of RSV-CAT, 2 pmol of test plasmid, and 2 μg of pSG5-p110-CAAX or the empty vector pSG5 (to adjust total plasmid quantity) were used. To study the effects of TSH and IGF-I, transfected cells were cultured in starvation medium (72 h) and then treated with 1 nM TSH and/or 100 ng/ml IGF-I. The PI3K inhibitor LY294002 was studied by addition at 10 μM, 30 min before hormone addition. After 24 h, cells were collected for LUC and CAT activity assay, as described previously (50, 51).

Acknowledgments

We are indebted to Dr. Nancy Carrasco (Albert Einstein College of Medicine, Bronx, NY) for the NIS cDNA and the NIS antibody, to Dr. Teresa Iglesias (Instituto de Investigaciones Biomédicas, Madrid, Spain) for the antiphospho-PKD antibody, and to Dr. Julian Downward (Imperial Cancer Research Foundation, London, UK) for the p110CAAX and p85$\alpha\Delta$iSH2-N expression vectors. We thank Catherine Mark for linguistic assistance.

Received March 26, 2001. Accepted October 29, 2001.

Address all correspondence and requests for reprints to: Dr. Pilar Santisteban, Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Arturo Duperier, 4, E-28029 Madrid, Spain. E-mail: psantisteban@iib.uam.es.

This work was supported by Dirección General de Investigación Cientifica y Técnica Grants PM97/0065 and BMC2001-2087 and Comunidad Autónoma de Madrid Grant 08.1/0025/97-99. B.G. is the recipient of a postdoctoral fellowship from the Comunidad Autónoma de Madrid.

REFERENCES

1. De la Vieja A, Dohan O, Levy O, Carrasco N 2000 Molecular analysis of the sodium/iodide symporter: impact on thyroid and extrathyroid pathophysiology. Physiol Rev 80:1083–1105
2. Dai G, Levy O, Carrasco N 1996 Cloning and characterization of the thyroid iodide transporter. Nature 379: 458–460
3. Weiss SJ, Philp NJ, Ambesi-Impiombato FS, Grollman EF 1984 Thyrotropin-stimulated iodide transport mediated by adenosine 3′,5′-monophosphate and dependent on protein synthesis. Endocrinology 114:1099–1107
4. Nissim M, Lee KO, Petrick PA, Dahlberg PA, Weintraub BD 1987 A sensitive thyrotropin (TSH) bioassay based on iodide uptake in rat FRTL-5 thyroid cells: comparison with the adenosine 3′,5′-monophosphate response to human serum TSH and enzymatically deglycosylated bovine and human TSH. Endocrinology 121:1278–1287
5. Roger PP, Dumont JE 1984 Factors controlling proliferation and differentiation of canine thyroid cells cultured in

reduced serum conditions: effects of thyrotropin, cyclic AMP and growth factors. Mol Cell Endocrinol 36:79–93
6. Levy O, Dai G, Riedel C, Ginter CS, Paul EM, Lebowitz AN, Carrasco N 1997 Characterization of the thyroid Na^+/I^- symporter with an anti-COOH terminus antibody. Proc Natl Acad Sci USA 94:5568–5573
7. Paire A, Bernier-Valentin F, Selmi-Ruby S, Rousset B 1997 Characterization of the rat thyroid iodide transporter using anti-peptide antibodies. Relationship between its expression and activity. J Biol Chem 272: 18245–18249
8. Kogai T, Endo T, Saito T, Miyazaki A, Kawaguchi A, Onaya T 1997 Regulation by thyroid-stimulating hormone of sodium/iodide symporter gene expression and protein levels in FRTL-5 cells. Endocrinology 138:2227–2232
9. Schmutzler C, Winzer R, Meissner-Weigl J, Köhrle J 1997 Retinoic acid increases sodium/iodide symporter mRNA levels in human thyroid cancer cell lines and suppresses expression of functional symporter in nontransformed FRTL-5 rat thyroid cells. Biochem Biophys Res Commun 240:832–838
10. Pekary AE, Hershman JM 1998 Tumor necrosis factor, ceramide, transforming growth factor-β1, and aging reduce Na^+/I^- symporter messenger ribonucleic acid levels in FRTL-5 cells. Endocrinology 139:703–712
11. Tong Q, Ryu KY, Jhiang SM 1997 Promoter characterization of the rat Na^+/I^- symporter gene. Biochem Biophys Res Commun 239:34–41
12. Ohno M, Zannini M, Levy O, Carrasco N, Di Lauro R 1999 The paired-domain transcription factor Pax8 binds to the upstream enhancer of the rat sodium/iodide symporter gene and participates in both thyroid-specific and cyclic-AMP-dependent transcription. Mol Cell Biol 19: 2051–2060
13. Ohmori M, Endo T, Harii N, Onaya T 1998 A novel thyroid transcription factor is essential for thyrotropin-induced up-regulation of Na^+/I^- symporter gene expression. Mol Endocrinol 12:727–736
14. Endo T, Kaneshige M, Nakazato M, Ohmori M, Harii N, Onaya T 1997 Thyroid transcription factor-1 activates the promoter activity of rat thyroid Na^+/I^- symporter gene. Mol Endocrinol 11:1747–1755
15. Myers MG, White MF 1996 Insulin signal transduction and the IRS proteins. Annu Rev Pharmacol Toxicol 36: 615–658
16. O'Brien RM, Granner DK 1996 Regulation of gene expression by insulin. Physiol Rev 76:1109–1161
17. Santisteban P, Kohn LD, Di Lauro R 1987 Thyroglobulin gene expression is regulated by insulin and insulin-like growth factor 1, as well as thyrotropin, in FRTL-5 thyroid cell. J Biol Chem 262:4048–4052
18. Zarrilli R, Formisano S, Di Jeso B 1990 Hormonal regulation of thyroid peroxidase in normal and transformed rat thyroid cells. Mol Endocrinol 4:39–45
19. Santisteban P, Acebron A, Polycarpou-Schwarz M, Di Lauro R 1992 Insulin and insulin-like growth factor I regulate a thyroid-specific nuclear protein that binds to the thyroglobulin promoter. Mol Endocrinol 6:1310–1317
20. Aza-Blanc P, Di Lauro R, Santisteban P 1993 Identification of a *cis*-regulatory element and a thyroid-specific nuclear factor mediating the hormonal regulation of rat thyroid peroxidase promoter activity. Mol Endocrinol 7:1297–1306
21. Civitareale D, Lonigro A, Sinclair J, Di Lauro R 1989 A thyroid-specific nuclear protein essential for the tissue specific expression of the thyroglobulin promoter. EMBO J 8:2537–2542
22. Francis-Lang H, Price M, Polycarpou-Schwarz M, Di Lauro R 1992 Cell-type-specific expression of the rat thyroperoxidase promoter indicates common mechanism for thyroid-specific gene expression. Mol Cell Biol 12:576–588
23. Ortiz L, Zannini M, Di Lauro R, and Santisteban P 1997 Transcriptional control of the forkhead thyroid transcription factor TTF-2 by thyrotropin, insulin, and insulin-like growth factor I. J Biol Chem 272:23334–23339
24. Saji M, Akamizu T, Sanchez M, Obici S, Avvedimento E, Gottesman ME, Kohn LD 1992 Regulation of thyrotropin receptor gene expression in rat FRTL-5 thyroid cells. Endocrinology 130:520–533
25. Porras A, Alvarez AM, Valladares A, Benito M 1998 p42/p44 mitogen-activated protein kinases activation is required for the insulin-like growth factor-I/insulin induced proliferation, but inhibits differentiation, in rat fetal brown adipocytes. Mol Endocrinol 12:825–834
26. Matthews SA, Rozengurt E, Cantrell D 1999 Characterization of serine 916 as an *in vivo* autophosphorylation site for protein kinase D/protein kinase C. J Biol Chem 274:26543–26549
27. Zacarija M, McKenzie JM 1989 Variations in the culture medium for FRTL-5 cells: effects on growth and iodide uptake. Endocrinology 125:1253–1259
28. Saji M, Kohn LD 1991 Insulin and insulin-like growth factor-I inhibit thyrotropin-increased iodide transport in serum-depleted FRTL-5 rat thyroid cells: modulation of adenosine 3′,5′-monophosphate signal action. Endocrinology 128:1136–1143
29. De Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL 1998 Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477
30. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM 1998 A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279
31. Dremier S, Vandeput F, Zwartkruis FJT, Bos JL, Dumont, JE, Maenhaut C 2000 Activation of the small G protein Rap1 in dog thyroid cells by both cAMP-dependent and -independent pathways. Biochem Biophys Res Commun 267:7–11
32. Tsygankova OM, Saavedra A, Rebhun JF, Quillian LA, Meinkoth JL 2001 Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. Mol Cell Biol 21:1921–1929
33. Cass LA, Summers SA, Prendegast GV, Backer JM, Birnbaum MJ, Meinkoth JL 1999 Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol 19:5882–5891
34. Medina DL, Toro MJ, Santisteban P 2000 Somatostatin interferes with thyrotropin-induced G1-S transition mediated by PKA and PI3-K: involvement of RhoA and cyclin E-CDK2 complexes. J Biol Chem 275: 15549–15556
35. Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT 1994 A novel phosphoinositide 3 kinase activity in myeloid-derived cell is activated by G protein β-γ subunits. Cell 77:83–93
36. Cass LA, Meinkoth JL 2000 Ras signaling through PI3K confers hormone-independent proliferation that is compatible with differentiation. Oncogene 19:924–932
37. Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD 1997 Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci 22: 267–272
38. Downward J 1998 Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10:262–267
39. Ringel MD, Hayre N, Saito J, Saunier B, Schuppert F, Burch H, Bernet V, Burman KD, Kohn LD, Saji M 2001 Overexpression and overactivation of akt in thyroid carcinoma. Cancer Res 61:6105–6111
40. Bruni P, Boccia A, Baldassarre G, Trapasso F, Santoro M, Chiappetta G, Fusco A, Viglietto G 2000 PTEN expression is reduced in a subset of sporadic thyroid carcinomas: evidence that PTEN-growth suppressing

activity in thyroid cancer cells mediated by p27kip1. Oncogene 19:3146–3155
41. Kops GJPL, Burgering BMT 1999 Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. J Mol Med 77:656–665
42. Ambesi-Impiombato FS, Parks LAM, Coon HG 1980 Culture of hormone-dependent functional cells of rat thyroid. Proc Natl Acad Sci USA 77:3455–3459
43. Kohn LD, Valente WA, Grollman EF, Aloj SM, Vitti P 1986 Clinical determination and/or quantification of thyrotropin and a variety of thyroid stimulatory or inhibitory factors performed *in vitro* with an improved cell line FRTL-5. US Patent 4:609–622
44. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
45. Kaminsky SM, Levy O, Salvador C, Dai G, Carrasco N 1994 Na^+-I-symport activity is present in membrane vesicles from thyrotropin-deprived non-I(−)-transporting cultured thyroid cells. Proc Natl Acad Sci USA 91: 3789–3793
46. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
47. Chen C, Okayama H 1988 Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. Biotechniques 6:632–639
48. Gorman CM, Merlino GT, Willingham MC, Pastan I, Howard BM 1982 The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukariotic cells by DNA-mediated transfection. Proc Natl Acad Sci USA 79:6777–6781
49. Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD, Downward J 1996 Activation by phosphoinositide 3-kinase by interaction with Ras and by point mutations. EMBO J 15:2442–2451
50. Gorman CM, Moffat LM, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044–1051
51. De Wet JR, Wood KV, De Luca M, Helinski DR, Subramani S 1987 The firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol 7:725–737